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ORIGINAL PAPER

Absence of caveolin-1 alters heat shock protein expression in spontaneous mammary tumors driven by Her-2/neu expression

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Abstract In a previous study, we measured caveolin-1 protein levels, both in the normal breast and in breast cancer. The study revealed no association between caveolin-1 expression in the epithelial compartment and clinical disease outcome. However, high levels of caveolin-1 in the stromal tissue surrounding the tumor associated strongly with reduced metastasis and improved survival. Using an animal model, we found that the onset of mammary tumors driven by Her-2/neu expression was accelerated in mice lacking caveolin-1. We have analysed the heat shock protein (Hsp) response in the tumors of mice lacking caveolin-1. In all cases, the mammary tumors were estrogen and progesterone receptor negative, and the levels of Her-2/neu (evaluated by immunohistochemistry) were not different between the caveolin-1 +/+ ($n = 8$) and the caveolin-1 −/− ($n = 7$) tumors. However, a significant reduction in the extent of apoptosis was observed in mammary tumors from animals lacking caveolin-1. While Bcl-2, Bax, and survivin levels in the tumors were not different, the amount of HSPA (Hsp70) was almost double in the caveolin-1 −/− tumors. In contrast, HSPB1 (Hsp27/Hsp25) levels were significantly lower in the caveolin-1 −/− tumors. The mammary tumors from caveolin-1 null mice expressed more HSPC4 (gp96 or grp94), but HSPC1 (Hsp90), HSPA5 (grp78), HSPD1 (Hsp60), and CHOP were not altered. No significant changes in these proteins were found in the stroma surrounding these tumors. These results demonstrate that the disruption of the Cav-1 gene can cause alterations of specific Hsps as well as tumor development.

Keywords Caveolin-1 · Heat shock proteins · Breast cancer · Her-2/neu · Oncogene

Abbreviations

ER	Estrogen receptor
HSF-1	Heat shock factor-1
Her-2/neu	c-erbB-2
Hsp	Heat shock protein
IHC	Immunohistochemistry
PCNA	Proliferating cell nuclear antigen
PR	Progesterone receptor

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Introduction

Caveolae are plasma membrane invaginations involved in molecular trafficking via endocytosis and transcytosis.

The main structural component of caveolae is a 22 kDa protein called caveolin-1 (Cav-1), which is also present in the cytosol. In physiological conditions, Cav-1 acts as a scaffolding protein that regulates lipid homeostasis, as well as the activity of several receptors and signalling molecules (Parton and Simons 2007). In oncology, this protein is involved in carcinogenesis, tumor invasion, and metastasis, but the consequences of its participation are variable, depending on the tumor type (Goetz et al. 2008). For example, in prostate cancer cells, Cav-1 is up-regulated by VEGF, TGF-β1, and FGF2, which in turn leads to increased levels of these factors (both at the transcript and protein levels), resulting in enhanced cell motility and migration (Li et al. 2009). On the other hand, in melanoma there is experimental evidence that Cav-1 may function as a metastasis suppressor, reducing the ability of B16F10 cells to form lung metastases (Trimmer et al. 2010).

In a recent study, we assessed Cav-1 levels in normal human breast and in breast cancer tissues (Sloan et al. 2009). In normal breast, Cav-1 was present in stromal fibroblasts and in myoepithelial cells but not in luminal epithelial cells, while in carcinomas, Cav-1 was expressed in the epithelial compartment of some tumors. This study revealed a lack of association between Cav-1 expression in the epithelial compartment and clinical outcome. In contrast, high levels of Cav-1 in stromal tissues surrounding the tumor associated strongly with reduced metastasis and improved survival (Sloan et al. 2009). In independent studies, a lack of stromal Cav-1 expression was confirmed as an important predictor for early tumor recurrence and poor clinical outcome in breast cancer patients (Witkiewicz et al. 2009a; Koo et al. 2011), and as the most important prognostic factor for poor overall survival in triple negative and basal-like breast cancer patients (Witkiewicz et al. 2010). Moreover, in patients with ductal carcinoma in situ, an absence of stromal Cav-1 was associated with early disease progression to invasive breast cancer, with reduced time to recurrence and a higher recurrence rate (Witkiewicz et al. 2009b). Transcript profiling of wild type and Cav-1 null fibroblasts revealed enhanced expression of genes associated with oxidative stress, leading to activation of HIF and NF-κB (Pavlides et al. 2010). This enhanced oxidative stress could, therefore, mimic hypoxia and drive inflammation in the tumor microenvironment. In humans, Cav-1 mutations are associated with lipodystrophy, cell transformation, and cancer while in mice disruption of the Cav-1 gene can cause cardiovascular diseases, diabetes, cancer, atherosclerosis, and pulmonary fibrosis (Bosch et al. 2011).

The onset of mammary tumors driven by mammary-specific expression of Her2/neu was accelerated in mice lacking Cav-1 (Sloan et al. 2009), thereby supporting the hypothesis that the presence of Cav-1 in the tumor microenvironment regulates the rate of tumor development. Since the loss of Cav-1 has been related to oxidative stress and the Hsp are stress related, the absence of Cav-1 is likely to initiate a stress response in cells.

The Hsp constitute a superfamily of molecular chaperones present in all cells and in all cell compartments, operating in a complex interplay with synergistic/overlapping multiplicity of functions, even though the common effect is cell protection (Ciocca and Calderwood 2005; Calderwood et al. 2006). Previous studies have linked Cav-1 with the Hsp response. For example, in breast cancer tissues, there are interactions between β-catenin, HSPB1 (also known as Hsp27/Hsp25, Kampinga et al. 2009), and Cav-1, as revealed by Western blot analysis and immunoprecipitation studies (Fanelli et al. 2008). Interactions between Cav-1 and HSPA/Hsp70 have been shown in the rat kidney under experimental conditions (Bocanegra et al. 2010). In human and mouse skin, Black et al. (2011) reported that the exposure to sulfur mustard caused up-regulation of Cav-1, Hsp27, and Hsp70, and that these proteins were localized in caveolae, hence supporting caveolae-mediated regulation of Hsp expression. Finally, caveola endocytosis is regulated by the actin cytoskeleton (Lajoie and Nabi 2010) and Hsp27 regulates actin polymerization (Doshi et al. 2009). Overall, these data illustrate the links between Cav-1 and Hsps and support our hypothesis that the loss of Cav-1 and the subsequent oxidative stress result in activation of a stress response in cells. In this study, we have completed a detailed immunohistochemical analysis of the effects of the loss of Cav-1 on the Hsp response in Her-2/neu expressing mammary tumors from Cav-1 null mice.

Materials and methods

Tumor bearing mice

Mice lacking Cav-1 and with mammary-specific expression of Her-2/neu were generated by crossing Cav-1 null mice (129/Sv/C57Bl/6) obtained from Dr. T. Kurzchalia (Drab et al. 2001) with mice transgenic for the MMTV-neu oncogene (Guy et al. 1992), as described previously (Sloan et al. 2009). Once the mammary tumors became palpable they were measured weekly using electronic callipers, and mice were culled when the primary tumor reached a volume of 1,500 mm^3. The tumors were removed at autopsy, weighed and fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. All procedures were performed in a barrier facility under protocols approved by the Peter MacCallum Animal Experimentation Ethics Committee.

Immunohistochemistry (IHC)

Hematoxylin and eosin stained tissue sections (5 µm thickness) were used for histopathological studies. Serial 5 µm-thick sections were mounted onto 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO)-coated slides for subsequent IHC analysis. The primary antibodies used in this study are described in Table 1. An antigen retrieval protocol using heat was used to unmask the antigens (30 min in citrate buffer 0.01 M, pH 6.0). Tissue sections were incubated with the primary antibodies overnight at 4°C in humidity chambers at the dilutions given in Table 1. A commercial kit to detect mouse and rabbit primary antibodies was used [Dako EnVision system, horse radish peroxidase, diaminobenzidine (DAB), from Dako, Carpinteria, CA]. Slides were lightly counterstained with hematoxylin to reveal nuclei, examined and photographed with a Nikon Eclipse E200 microscope (Japan). Non-specific mouse IgG1 antibody and purified rabbit pre-immune serum (DAKO, Kingsgrove, NSW, Australia) were used as isotype negative controls. As positive controls, we used breast cancer tissue blocks from our tumor bank (Sloan et al. 2009), the optimal dilution of each antibody was tested using these samples. The immunostaining was evaluated in the whole sections with the extent and intensity of immunostaining assessed independently by two experienced researchers blinded to the Cav-1 status of the sample. Disagreements (<10%, often relating to the level of staining intensity) were resolved by consensus. We used a scoring system reported previously (Gago et al. 1998). Briefly, we used an intensity score: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining and a proportion score: 0 = no staining, 1 = staining in less than 1/10 of the cells, 2 = 1/10 to <1/3 of the cells, 3 = 1/3 to <2/3 of the cells, and 4 = >2/3 of the cells. The total was obtained by combining both intensity and proportion scores, which were reported for membrane, cytoplasmic, and nuclear cell compartments. For PCNA, the percentage of positive nuclei was obtained based on an average of 700 cells counted per sample, at 400× magnification.

Apoptosis

Apoptosis was measured by the modification of the TUNEL assay using the ApopTag Plus in situ detection kit (Oncor, Gaithersburg, Maryland), as reported previously (Cuello-Carrión and Ciocca 1999). The apoptotic index (AI) was calculated as the percentage of positive nuclei, based on an average of 700 cells counted per case, at 400× magnification. Massive apoptosis was also evaluated in H&E stained tissues under the microscope by an image analyzer (*Y* axis in the graph represents relative unit area).

Statistical analysis

Statistical analyses were completed using the Prism computer program (Graph Pad Software, San Diego, CA); two-tailed paired *t* test was used for data analysis, a $p < 0.05$ was considered significant.

Table 1 Main characteristics of the antibodies used in the present study

Antibody	Source	Dilution
Her-2/neu	RAb (Dako, Glostrup, Denmark)	1:200
Cav-1[a]	RAb (BD Transduction Lab, Pharmingen, San Diego, CA)	1:500
ERα[a]	MAb (clone 1D5, Dako)	1:100
ERβ[a]	MAb (Novocastra-Leica, Wetzlar, Germany)	1:50
PR[a]	MAb (clone 636, Dako)	1:80
p53[a]	MAb (clone DO-7, Dako)	1:70
Bcl-2[a]	MAb (clone 124, Dako)	1:80
Bax[a]	MAb (MBL, Japan)	1:250
Survivin[a]	RAb (Oncogene, CN Biosciences Co. La Jolla, CA)	1:200
PCNA[a]	MAb (clone PC-10, Dako)	1:700
HSF-1[a]	RAb (Stressgen, Victoria, Canada)	1:1,000
Hsp27	RAb, against hybrid Hsp25/27[b]	1:1,000
Hsp60[a]	MAb (clone LK2, Sigma Chemical Co, St. Louis, MO)	1:100
Hsp70[a]	MAb (clone BRM-22, Sigma)	1:1,000
Grp78[a]	RAb (Stressgen)	1:200
Hsp90[a]	MAb (clone AC88, Stressgen)	50 µg/ml
Gp96[a]	RMAb (9G10.F8.2, NeoMarkers, Freemont, CA)	1:100
CHOP[a]	RAb (gift, Dr. LM Hendershot, Memphis, TN)	1:100

RAb rabbit antibody (polyclonal), *MAb* mouse monoclonal antibody, *RMAb* rat monoclonal antibody

[a] The antigen retrieval protocol (see "Materials and methods" section) was used with this antibody

[b] Data on this antibody have been published elsewhere (Fanelli et al. 2008)

Results

Main characteristics of the Cav-1 +/+ tumors driven by Her-2/neu expression

The tumors exhibit an organoid growth pattern, forming large groups of cancer cells with cytologic anaplasia. These tumors frequently showed comedo-type massive apoptosis in the central tumor nests (Fig. 1a). Although the tumors presented a clear limit with the surrounding normal mammary tissues, they were clearly invasive in connective and muscle tissues and lung metastases were reported previously (Sloan et al. 2009). In few cases there were areas of tumor with mixed papillary and mucinous characteristics. The stroma between the large groups of cancer cells showed numerous fibroblasts, good vascularization, with no obvious signs of inflammation (lymphoid infiltrate).

IHC revealed high levels of Her-2/neu oncoprotein at the cell membrane, with the immunoreactivity more clearly noted in cells located at the periphery of the tumor nests (Fig. 1b). As expected, the "normal" mammary duct epithelial cells located close to the tumor areas showed nuclear ERα expression, but the tumor cells showed complete absence of ERα (Fig. 1c). The tumor cells also lacked ERβ and PR (data not shown) while the PCNA immunostaining revealed a relatively high proliferation rate (33.17%). Finally, Cav-1 was expressed in the stroma (adipose cells, some fibroblasts, and endothelial cells) but not in the tumor cells (Fig. 1d).

Effects of the absence of Cav-1 on the tumors

The absence of Cav-1 in the Cav-1 KO tumors was confirmed by IHC (Fig. 1e). The protein levels of Her-2/neu (score 4.75 vs. 4.70% in the Cav-1 +/+ and Cav-1 −/−, respectively) and that of PCNA (33.17 vs. 31.05%) were not significantly affected by the absence of Cav-1, and the tumors remained negative for ERα, ERβ, and PR (data not shown). Although the absence of Cav-1 did not affect the growth pattern of the tumors, it did significantly decrease the amount of apoptosis, assessed by H&E staining (Fig. 1f) and by TUNEL staining ($p < 0.03$) (Fig. 1g–h and graph), resulting in tumors with a cribriform growth pattern (Fig. 1f).

Tumor expression of proteins involved in apoptosis and stress responses

To see if the difference in the extent of apoptosis was correlated with some of the genes involved in apoptosis, the tumors were immunostained for Bcl-2, Bax, and p53, but no differences were noted (data not shown). p53 was not mutated or inactivated as evidenced by the lack of immunostaining in all of the samples tested. While survivin protein levels were low in both groups, there was a non-significant trend toward higher levels in the tumors grown in Cav-1 KO mice (score 1.85 vs. 1.12 for Cav-1 +/+ tumors).

Previous studies have shown that certain Hsps such as Hsp27 and Hsp70 are involved in apoptosis resistance (Garrido et al. 2006). We evaluated these two proteins and found a significant increase in Hsp70 levels (4.86 vs. 2.62,

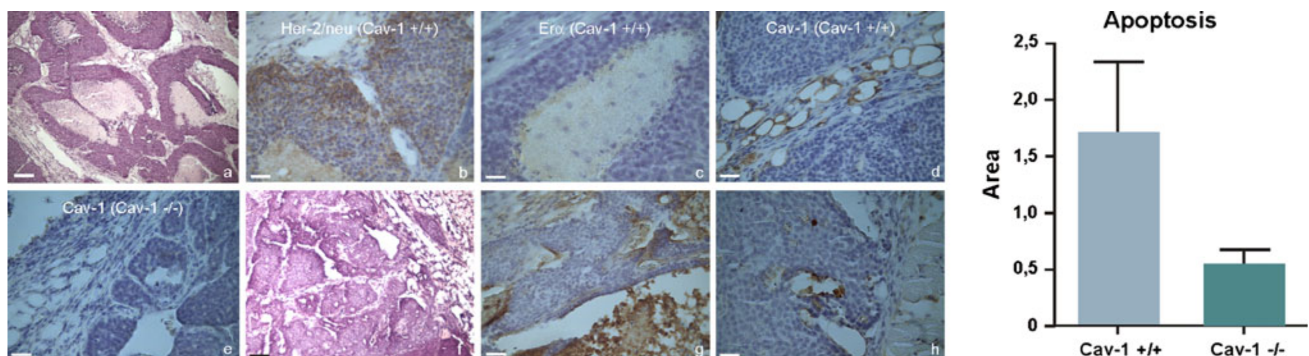

Fig. 1 Representative photographs with the main histological characteristics of the mammary tumors. Large areas of massive apoptosis can be seen in a tumor from a Cav-1 +/+ mouse (**a**, hematoxylin and eosin staining). IHC revealed that these tumors display high levels of Her-2/neu at the cell membrane (**b**), that they lacked of ERα (**c**), and that Cav-1 was present in the stroma but not in the tumor cells (**d**). In contrast, in the mammary tumors from Cav-1 −/− mice (**e**, note the lack of Cav-1 as revealed by IHC), a significant decrease in massive apoptosis was noted (**f**, hematoxylin and eosin staining). TUNEL staining revealed numerous apoptotic cells in the central areas of the tumor nests in the Cav-1 +/+ animals (**g**), and fewer in the tumors arising in the Cav-1 −/− mice (**h**). The graph shows quantitation of the extent of apoptosis (*Y* axis: relative unit area) in the tumors from Cav-1 +/+ and Cav-1 −/− mice. Note that in this study we did not have background cross-reaction problems with the immune detection of antigens even when some of the antibodies were of mouse origin (Table 1) for use in a mouse animal model (**c**). The images were captured with a Nikon Eclipse E200 microscope (×10–60 objectives). The positive immunoreactivity appears as brown deposits, and the slides were lightly counterstained with hematoxylin to reveal nuclei. Figure magnifications, **a** *bar* 230 µm, **b** *bar* 90 µm, **c** *bar* 80 µm, **d** *bar* 120 µm, **e** *bar* 90 µm, **f** *bar* 200 µm, **g** *bar* 100 µm, **h** *bar* 90 µm

$p = 0.03$) in the tumor cells grown in Cav-1 KO mice (Fig. 2a–c). In contrast, Hsp27 was significantly decreased (1.42 vs. 3.25, $p = 0.03$) in the tumors grown in Cav-1 deficient mice (Fig. 2d–f). In both Cav-1 +/+ and null tumors, Hsp27 levels were higher in the tumor cells located closest to the areas of massive apoptosis. Gp96 protein levels increased in the tumors grown in Cav-1 KO mice (Fig. 2e, f) but the levels were relatively low (2.3 vs. 1.5, not statistically significant). The levels of HSF-1 and of the other Hsps examined (Hsp60, Grp78, Hsp90, and CHOP) were not changed significantly by the lack of Cav-1 (Table 2).

Effects of the absence of Cav-1 on protein levels in the stroma

Although we paid particular attention to the cell composition, lymphoid and macrophage infiltration, sclerosis, vascularization, proliferation, apoptosis, and to the expression levels of the protein markers under evaluation, no significant changes were noted in stromal cells in the tumor microenvironment.

Discussion

In this study, we report that the absence of Cav-1 alters the expression of certain Hsp in the tumor cells, but not in the stroma. Although significant, the changes were not dramatic, with small changes observed in Hsp70 and Hsp27 and even less in Gp96. While it is generally accepted that Cav-1 is essential for the formation of caveolae, a recent study has shown by electron microscopy that intestinal cells may still form large size caveolae in the absence of Cav-1 (Cipriani et al. 2011), indicating that these cells have

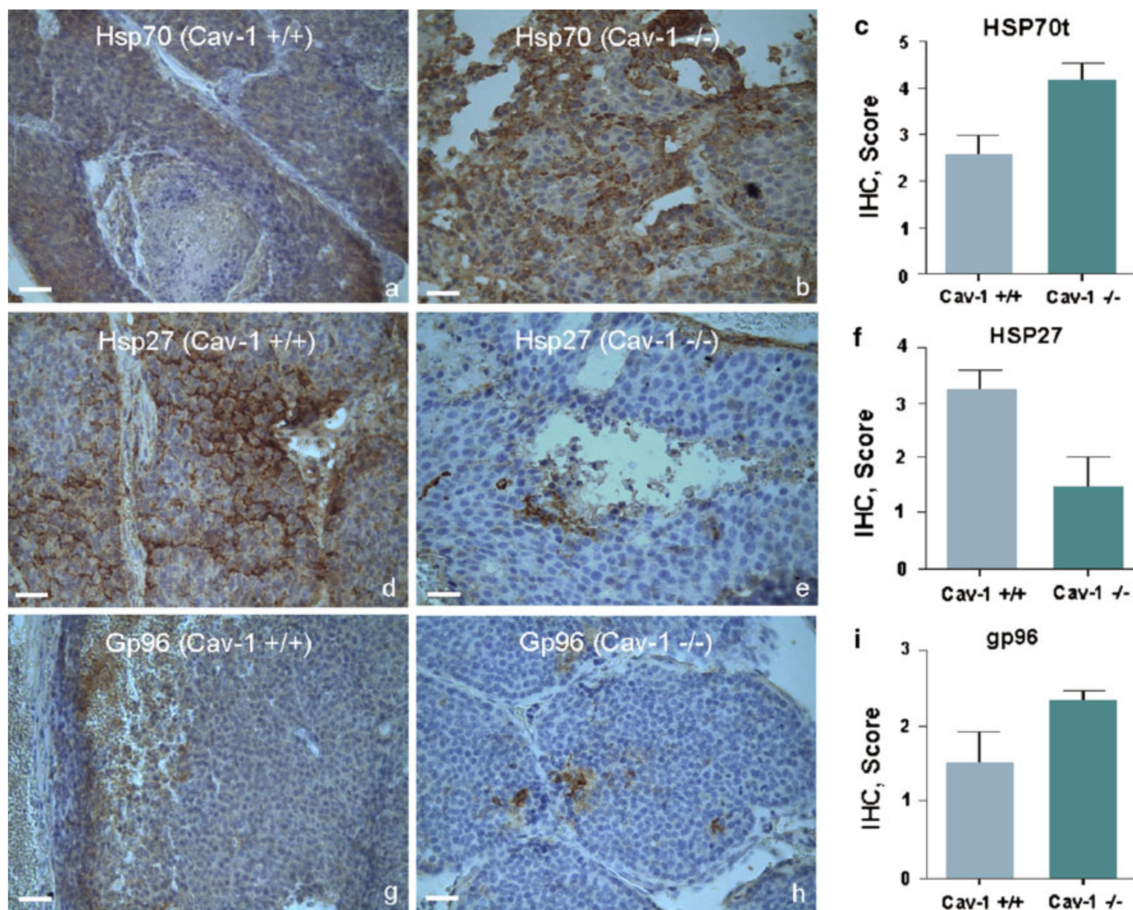

Fig. 2 Representative images of the levels of Hsp70, Hsp27, and Gp96 as revealed by IHC. Hsp70 shows weak immunostaining intensity in tumors grown in a Cav-1 +/+ mouse (**a**), but shows strong immunostaining intensity in the cytoplasm of the tumor cells from a Cav-1 null mouse (**b**). The difference in the scores is shown graphically (**c**). The BRM-22 antibody detects both the constitutive and inducible Hsp70 (Hsp70t). Hsp27 shows strong immunostaining intensity at the cell membrane of the tumors removed from a Cav-1 +/+ mouse (**d**). In contrast, the protein is expressed in only a few tumor cells in a tumor from a Cav-1 KO mouse (**e**). Note Hsp27 expression in the stroma as well (*right upper corner*) (**e**). The difference in the Hsp27 scores is shown graphically (**f**). Gp96 is absent in tumors from a Cav-1 +/+ mouse (**g**), but is apparent in a few tumor cells from a Cav-1 null mouse (**h**). The difference in Gp96 levels was not significant (**i**). The images were captured with a Nikon Eclipse E200 microscope (×10–40 objectives). Figure magnifications, **a** *bar* 120 μm, **b** *bar* 120 μm, **d** *bar* 90 μm, **e** *bar* 100 μm, **g** *bar* 120 μm, **h** *bar* 120 μm

Table 2 IHC evaluation of HSF-1 and Hsp in the tumors

Molecule	Score[a] in Cav-1 +/+	Score in Cav-1 −/−	*p* value
HSF-1	0.25	0.28	NS
Hsp60	0	0	NS
Grp78	3.75	3.85	NS
Hsp90	1.65	1.50	NS
CHOP	1.5	1.70	NS

NS not significant

[a] The scoring system used was described in the "Materials and methods" section

other proteins to circumvent the lack of Cav-1. However, the absence of Cav-1 has important biological consequences, confirmed by the shorter tumor onset time and shorter overall survival of mice transgenic for the MMTV-neu oncogene (Sloan et al. 2009) and the MMTV-PyMT viral oncogene (Williams et al. 2004).

We report that the lack of Cav-1 does not alter proliferation of mammary tumors but significantly decreases the extent of apoptosis. This is consistent with a previous study where Cav-1-null mice have been used as an animal model to study hyperoxia-induced apoptosis (Jin et al. 2008; Zhang et al. 2009). These authors examined lung tissues and fibroblasts from Cav-1 −/− mice and found increased cytoprotection following exposure to high O_2 tension, via up-regulation of heme oxygenase-1 and survivin. In our study, we saw a trend toward increased survivin in the Cav-1 null tumors, but not a significant change. The lack of frozen tissues prevents us from complementing the IHC analysis with more quantitative methods like Western blotting. However, our results suggest that the contribution of survivin to apoptosis resistance in Cav-1 null tumors might be complemented by the higher expression of Hsp70, a well-known cytoprotective protein. Hsp70 has been reported to prevent the release of cytochrome *c* from the mitochondria (Steel et al. 2004) most likely by inhibiting the translocation of Bax to the mitochondria (Stankiewicz et al. 2005). Hsp70 has also been shown to inhibit heat-induced apoptosis upstream of the mitochondria by preventing Bax translocation. (Cheng et al. 2011). High expression of Hsp70 contributes (along with other Hsps) to p53 stabilization and is related to disease prognosis in classical Hodgkin lymphoma (Santón et al. 2011). Other proteins also contribute to apoptosis resistance in this tumor model. Castello-Cros et al. (2011) reported that the stromal fibroblasts lacking Cav-1 significantly increased plasminogen activator inhibitor type 1 and type 2 (PAI-1 and PAI-2) expression and that in xenografts, co-inoculation with fibroblasts that stably express either PAI-1 or PAI-2 significantly reduced the extent of apoptosis of MDA-MB-231 human breast tumors. Finally, we cannot rule out the possibility that Hsp70 is also playing another role apart from the suppression of apoptosis.

In contrast to Hsp70, we found lower Hsp27 levels in the tumors grown in absence of Cav-1. Hsp27 can also regulate apoptosis by preventing apoptosome formation and the subsequent activation of caspases (Garrido et al. 2006). A decrease in the migratory capacity of the 4T1 murine mammary tumor cells was observed when Hsp25/Hsp27 was downregulated by siRNA (Bausero et al. 2006). Of interest in our study is that the group of animals with mammary tumors grown in Cav-1 null mice had a lower incidence of visible lung metastases than the tumors grown in Cav-1 +/+ mice (20 vs. 28%, Sloan et al. 2009). Potentially this could be explained by the lower tumor Hsp27 content revealed in the present study. This is consistent with a previous communication where Hsp27 was reported to interact with proteins involved with the cadherin–catenin cell adhesion system that is required for both cell–cell adhesion and downstream Wnt/wingless signaling pathways involved in cell invasion (Fanelli et al. 2008). Consistent with this, Hu et al. (2010) have reported that the hepatocyte growth factor downregulates E-cadherin, β-catenin, and Cav-1 in human ovarian cancer cells, leading to a disassembly of cell–cell contacts and an enhancement of their invasive and migratory properties.

The breast tumors analysed in this study are hormone independent, as shown by the lack of ER and PR. The relatively low levels of Hsp27 may be the consequence of this lack of hormone responsiveness since Hsp27 is estrogen regulated (Ciocca et al. 1997). The tumors were initiated by high Her-2/neu expression, and in humans we know that these tumors frequently lack or have low ER/PR and are usually resistant to hormone therapy (Ciocca et al. 2006). Furthermore, the binding of heregulin, to its receptor c-erbB3 and subsequent activation of Her-2/neu, causes activation of HSF-1, which binds to the co-repressor metastasis-associated protein-1 (MTA1) (Khaleque et al. 2008). This complex participates in the repression of estrogen-regulated promoters causing the repression of several genes including the pro-apoptotic c-Myc. In this study, we did not find significant changes in HSF-1 expression in the tumors grown in Cav-1 null mice, however, a more sensitive technique such as RT-PCR is needed to confirm this finding. Another important link is the interaction of Her-2/neu oncoprotein with Hsp27. In human breast cancer cell lines, Hsp27 increases Her-2/neu stability, thereby reducing susceptibility to trastuzumab (Kang et al. 2008). The tumors examined here were induced by Her-2/neu, and the lack of Cav-1 did not alter the expression of Her-2/neu, suggesting that the decrease in Hsp27 did not increase the turnover of the Her-2/neu oncoprotein.

In summary, we have shown that several stress related proteins are altered by loss of Cav-1 and that these changes

may contribute to the reduced apoptosis and earlier onset of mammary tumors that has been described in two different transgenic mouse models of breast cancer.

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